Macrophage immunomodulatory activity of polysaccharides isolated from *Opuntia polyacantha*

Igor A. Schepetkin\textsuperscript{a}, Gang Xie\textsuperscript{a}, Liliya N. Kirpotina\textsuperscript{a}, Robyn A. Klein\textsuperscript{b}, Mark A. Jutila\textsuperscript{a}, Mark T. Quinn\textsuperscript{a,⁎}

\textsuperscript{a} Department of Veterinary Molecular Biology, Montana State University, Bozeman, MT 59717, United States  
\textsuperscript{b} Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, United States

Received 26 March 2008; received in revised form 14 May 2008; accepted 6 June 2008

Abstract

*Opuntia polyacantha* (prickly pear cactus) has been used extensively for its nutritional properties; however, less is known regarding medicinal properties of *Opuntia* tissues. In the present study, we extracted polysaccharides from *O. polyacantha* and used size-exclusion chromatography to fractionate the crude polysaccharides into four polysaccharide fractions (designated as *Opuntia* polysaccharides C-I to C-IV). The average \( M_r \) of fractions C-I through C-IV was estimated to be 733, 550, 310, and 168 kDa, respectively, and sugar composition analysis revealed that *Opuntia* polysaccharides consisted primarily of galactose, galacturonic acid, xylose, arabinose, and rhamnose. Analysis of the effects of *Opuntia* polysaccharides on human and murine macrophages demonstrated that all four fractions had potent immunomodulatory activity, inducing production of reactive oxygen species, nitric oxide, tumor necrosis factor \( \alpha \), and interleukin 6. Furthermore, modulation of macrophage function by *Opuntia* polysaccharides was mediated, at least in part, through activation of nuclear factor \( \kappa B \). Together, our results provide a molecular basis to explain a portion of the beneficial therapeutic properties of extracts from *O. polyacantha* and support the concept of using *Opuntia* polysaccharides as an immunotherapeutic adjuvant.

© 2008 Elsevier B.V. All rights reserved.

1. Introduction

Stimulation of the innate immune system with immunomodulators can increase host resistance to unforeseen pathogenic threats [1], and a number of innate system immunomodulators have been identified, including cytokines [2], substances isolated from microorganisms and fungi [3], and substances isolated from plants [4,5]. Indeed, a wide range of bioactive polysaccharides have been isolated from various medicinal plants, and these polysaccharides have been shown to possess immunomodulatory activity through their ability to modulate macrophage function (reviewed in [6]). Appropriate enhancement of innate immune functions by bioactive compounds can then augment host defense responsiveness [7]. Thus, because of their low toxicity and high potency, plant polysaccharides...
represent ideal candidates for therapeutics with immunomodulatory action.

Although the prickly pear cactus, *Opuntia polyacantha* (subfamily Opuntioideae, family Cactaceae), and other *Opuntia* species are currently consumed for their nutritional properties [8,9], tissues from these cacti have been used traditionally in the past for their pharmacological properties [8,9]. For example, fruits and stems of many *Opuntia* species have been used in folk medicine for burns, wounds, edema, bronchial asthma, hypertension, indigestion, and type II diabetes (reviewed in [10,11]). Extracts of fruits and stems from *Opuntia* species have been reported to exhibit hypoglycemic [12], antiulcer [13], antioxidant [14], anti-inflammatory activity [15], hepatoprotective [16], and neuroprotective [17] activities. While glycoproteins and low-molecular weight compounds, such as flavonoids, proanthocyanidins, carotenoids, betalains, beta-sterols, and alpha-pyrones have been suggested to be among the active principles of *Opuntia* species [13,15,18–22], little is known regarding the medicinal properties of polysaccharides from *Opuntia*, and essentially nothing is known regarding their potential immunomodulatory properties. Indeed, the main substance produced by these plants is mucilage composed primarily of water and polysaccharides, which helps prevent dehydration and freezing of their tissues.

Among the numerous *Opuntia* species, bioactive polysaccharides have been isolated and characterized primarily from one member, *Opuntia ficus-indica* [12,23–26]. Polysaccharides from this species have been reported to exhibit hypoglycemic activity [12] and enhance re-epithelization in a model of dermal wounding [25]. Although macrophages play an important role in cutaneous wound healing [26], the potential immunomodulatory effects of polysaccharides from *Opuntia* in this process were not evaluated. Based on previous studies showing that many plant polysaccharides enhance immune function by activation of macrophages (reviewed in [6]), we suggest that at least part of the effects of *Opuntia* polysaccharides on wound healing is through modulation of macrophage function. Thus, it is now important to address this question and further define the contribution of polysaccharides to the therapeutic properties of *Opuntia* tissues.

In the present study, we extracted polysaccharides from *O. polyacantha* and isolated four polysaccharide fractions (designated as C-I to C-IV). Analysis of immunomodulatory effects on murine and human monocyte/macrophages demonstrated that *Opuntia* polysaccharides activated the production of nitric oxide (NO), reactive oxygen species, and cytokines, and stimulated nuclear factor \(\alpha\B) (NF-\alpha\B). The ability of *O. polyacantha* polysaccharides to modulate macrophage function suggests they may contribute to the therapeutic potential of tissues derived from this plant species.

2. Materials and methods

2.1. Reagents

\(\beta\)-glucosyl Yariv reagent [1,3,5-tri-(4-\(\beta\)-d-glucosyranosyl-oxynphenyl-azo)-2,4,6-trihydroxybenzene] was from Biosupplies Australia (Parkville, Australia). Gum Arabic was from Fluka BioChemica (Buchs, Switzerland) and 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione (L-012) was from Wako Chemicals (Richmond, VA). Sepharose 6B, galacturonic acid, galactose, arabinose, rhamnose, xylose, diphenylamine, aniline, anthrone, thiourea, trifluoroacetic acid (TFA), N-(1-naphthyl)ethylenediamine, sulfanilamide, phorbol-12-myristate-13-acetate (PMA), horseradish peroxidase, and lipopolysaccharide (LPS) from *Escherichia coli K-235* were from Sigma Chemical Co. (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM) and endotoxin-free fetal bovine serum (FBS) were from Mediatech Inc., Herndon, VA). Human granulocyte macrophage colony-stimulating factor (GM-CSF) was from Calbiochem (San Diego, CA).

2.2. Purification and fractionation of polysaccharides

Stems of cactus *O. polyacantha* were collected near Big Timber, MT. The fresh *O. polyacantha* stems (1.8 kg) were washed with distilled H\(_2\)O, homogenized, extracted with 6 l distilled H\(_2\)O, and the aqueous extract was centrifuged at 2000 ×g for 15 min. A four-fold volume of ethanol was added to the supernatant to precipitate polysaccharides overnight at 4 °C. The precipitate was pelleted by centrifugation, redissolved in distilled H\(_2\)O, sonicated for 10 min, and centrifuged at 11,000 ×g for 1 h. The supernatant was then filtered through a 0.22 \(\mu\)m filter and concentrated in an Amicon concentrator with a 5 kDa PMS membrane to obtain the crude polysaccharide extract (yield of 0.4% by weight).

The crude polysaccharide extract was fractionated by size-exclusion chromatography (SEC) on a Sepharose 6B column (2.5 × 92 cm) equilibrated with 0.01 M Tris – HCl buffer (pH 7.0) containing 0.15 M NaCl and eluted with the same buffer at a flow rate of 21 ml/h. The carbohydrate elution profile was determined by the phenol–H\(_2\)SO\(_4\) method, and absorbance was measured at 488 nm using a SpectraMax Plus microplate reader (Molecular Devices, Palo Alto, CA). The relevant fractions were pooled, concentrated, and lyophilized. For analysis of biological activity, the fractions were diluted in Hank’s balanced salt solution (HBSS) at the indicated concentrations (w/v) and filtered through sterile 0.22 \(\mu\)m filters.

2.3. Characterization of polysaccharide fractions

The pooled polysaccharide fractions were analyzed for protein content using a modified Lowry assay with bovine serum albumin as a standard, and carbohydrate content was determined by the phenol–H\(_2\)SO\(_4\) method with glucose as a standard. For nuclear magnetic resonance (NMR) analysis, samples (5 mg) were dissolved in deuterium oxide (0.5 ml), and \(^1\)H NMR spectra were recorded on a Bruker DRX-600 spectrometer (Bruker BioSpin, Billerica, MA) at 20 °C using 3-(trimethylsilyl)-propionic 2,2,3,3-,d\(_4\) acid sodium salt as an internal reference (δ 0.00 ppm).

The homogeneity and average \(M\) of the polysaccharide fractions were determined by high performance size-exclusion chromatography (HP-SEC) using a Shimadzu Class VP HPLC and Shodex OHpak SB-804 HQ column (8 mm × 300 mm) eluted with 50 mM sodium citrate buffer, pH 7.5, containing 0.15 M NaCl and 0.01% NaN\(_3\) at a flow rate of 0.3 ml/min. Peaks were detected using a refractive index detector (RID-10A; Shimadzu, Torrance, CA). Average \(M\) of the *Opuntia*
polysaccharide fractions were estimated by comparison with retention times of pullulan standards P-800, 400, 200, 100, 50 and 20 (Phenomenex, Torrance, CA), which have M_r of 788, 404, 212, 112, 47.3, and 22.8 kDa, respectively. Reproducibility of the retention times was typically >98%.

The presence of arabinogalactan in the samples was detected by single radial gel diffusion in 1% agarose gels containing 100 μg/ml β-glucosyl Yariv reagent, which selectively interacts with and precipitates compounds containing type II arabinogalactan structures. Four microliters of polysaccharide samples (10 mg/ml; w/v) were loaded into the wells, and the samples were incubated at 25 °C for 24 h in a humid atmosphere. A positive reaction was identified by a reddish circle around the well, and arabic gum (4 mg/ml) served as a positive control.

For monosaccharide composition analysis, samples were hydrolyzed at 100 °C for 6 h with 3 M TFA, and the resulting samples were separated by thin-layer chromatography (TLC) on Whatman silica gel 60 plates using monosaccharide standards for reference. The plates were developed with butanol/acetic acid/water (3:1:1), and bands were visualized by spraying the plates with aniline–diphenylamine reagent (2% aniline, 2% diphenylamine, and 8.5% H₃PO₄ acid in acetone) and heating at 100 °C for 10 min. Individual monosaccharide bands were scraped off the plate, extracted with H₂O₂, and quantified using a colorimetric method with monosaccharide standards. Briefly, the extracts were mixed with anithrone reagent (0.2% anthrone and 1% thiourea in H₂SO₄). After heating at 100 °C for 10 min, absorbance was measured at 620 nm.

2.4. Endotoxin assays

A Limulus Amebocyte Lysate (LAL) assay kit (Lonza, Walkersville, MD) was used to evaluate Opuntia polysaccharide samples for possible LPS contamination. To further evaluate the possible role of endotoxin, Opuntia polysaccharide fraction C-I was applied to a column containing Detoxi-Gel Endotoxin Removing Gel (Pierce, St. Louis, MO) and eluted with 0.05 M phosphate buffer containing 0.5 M NaCl to decrease ionic interactions of sample molecules with the affinity ligand. The concentration of polysaccharides in the eluted sample was adjusted to match that of the untreated fraction, as determined by the phenol–H₂SO₄ method, and both treated and untreated samples were analyzed for biological activity, as described below.

2.5. Cell cultures

Murine macrophage J774.A1 cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated, endotoxin-free FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin. Cells were grown to confluence in sterile tissue culture flasks and gently detached by scraping. Human monocyte-macrophage MonoMac6 cells (DSMZ, Germany) were grown in RPMI 1640 supplemented with 10% (v/v) endotoxin-free FBS, 10 μg/ml bovine insulin, 100 μg/ml streptomycin, and 100 U/ml penicillin. Human mononcytic THP1-Blue cells obtained from InvivoGen (San Diego, CA) were cultured in RPMI 1640 medium supplemented with 10% (v/v) endotoxin-free FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, 100 μg/ml zeocin, and 10 μg/ml blasticidin S. These cells are stably transfected with a secreted embryonic alkaline phosphatase gene that is under the control of a promoter inducible by nuclear factor κB (NF-κB).

All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. Cell number and viability were assessed microscopically using trypan blue exclusion.

2.6. Human monocytes-derived macrophages

Blood was collected from healthy donors in accordance with a protocol approved by the Institutional Review Board at Montana State University. Mononuclear cells were purified from the blood using dextran sedimentation, followed by Histopaque 1077 gradient separation and hypotonic lysis of red blood cells, as described previously [28]. Adherent monocytes were cultured for 7 days in RPMI-1640 containing 10% endotoxin-free FBS and 50 ng/ml GM-CSF to induce macrophage differentiation. Optimal conditions were maintained by refreshing the medium and cytokines every 3 days.

2.7. Analysis of NO production

J774.A1 cells were plated at a density of 1.5 × 10⁵ cells/well in a final volume of 200 μl in 96-well flat-bottom tissue culture plates and incubated in medium alone or medium containing various concentrations of polysaccharide fractions or E. coli LPS as a positive control. Cells were incubated at 37 °C in the presence of 5% CO₂ for 24 h, and 100 μl of the cell culture supernatants were removed and analyzed for NO using a colorimetric method with NaNO₂ as the standard. Briefly, supernatants were mixed with an equal volume of Griess reagent, which was prepared by mixing one part of 0.1% (w/v) N-(1-naphthyl)ethylenediamine with one part of 1% (w/v) sulfanilamide in 5% phosphoric acid. After 20 min, absorbance was measured at 540 nm using a SpectraMax Plus microplate reader.

2.8. Analysis of reactive oxygen species (ROS) production

Macrophage ROS production was analyzed using the chemiluminescent probe, L-012, which is highly sensitive for ROS generated in biologically complex systems. Murine J774.A1 macrophages (1.5 × 10⁵ cells/well) were incubated with various concentrations of polysaccharide fractions or positive control (LPS) for 24 h. After incubation, the culture supernatant was removed and replaced by an equal volume of HBSS supplemented with 25 μM L-012 and 5 μg/ml horseradish peroxidase with or without 100 nM PMA, as described previously [29]. Reactions were monitored on a Fluoroscan Ascent FL microtiter plate reader (ThermoElectron, Milford, MA) at 37 °C. Chemiluminescence was measured every 2 min for 2 h and is expressed as the integrated response over this time (arbitrary units).

2.9. Determination of TNF-α and IL-6

Cells were incubated for 24 h in culture media supplemented with 3% (v/v) endotoxin-free FBS with or without Opuntia polysaccharide fractions or LPS as a positive control in 96-well
plates at a density of $2 \times 10^5$ cells/well. Human monocyte-derived macrophages were plated in 96-well plates at a density of $6 \times 10^4$ cells/well. Human and murine TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences Pharmigen) were used to detect TNF-α/IL-6 in the cell supernatants. Cytokine concentrations were determined by extrapolation from TNF-α/IL-6 standard curves, according to the manufacturer's protocol.

### 2.10. Analysis of NF-κB activation

Activation of NF-κB was measured using an alkaline phosphatase reporter gene assay in THP1-Blue human monocytic cells. One week before an experiment, the cells were pre-activated overnight with PMA (50 ng/ml) to induce differentiation and plated in 96-well plates at a cell density of $2 \times 10^5$ cells/well. The cells were then incubated for 5 days with daily media changes. On day 7, *Opuntia* polysaccharide fractions or LPS (100 ng/ml) were added, the cells were incubated for 24 h, and alkaline phosphatase activity was measured in cell supernatants using QUANTI-Blue mix (InvivoGen, San Diego, CA). Activation of NF-κB is reported as an absorbance at 655 nm and compared with positive control samples (LPS).

### 2.11. Cytotoxicity assay

Cytotoxicity was analyzed with a CellTiter-Glo Luminescent Cell Viability Assay Kit (Promega, Inc., Madison, WI) according to the manufacturer's protocol. Briefly, cells were cultured at a density of $3 \times 10^4$ cells/well with the polysaccharide fractions or LPS (100 ng/ml) were added, the cells were incubated for 24 h, and alkaline phosphatase activity was measured in cell supernatants using QUANTI-Blue mix (InvivoGen, San Diego, CA). Activation of NF-κB is reported as an absorbance at 655 nm and compared with positive control samples (LPS).

### 2.12. Statistical analysis

Two-way analysis of variance (ANOVA) was performed on the indicated sets of data (GraphPad Prism Software, San Diego, CA). Pair-wise comparisons with differences at $P<0.05$ were considered to be statistically significant.

### 3. Results

#### 3.1. Preparation and characterization of *Opuntia* polysaccharides

Polysaccharides from *O. polycantha* were fractionated by preparative Sepharose 6B size-exclusion chromatography to obtain four main fractions, which were selected based on the total carbohydrate elution profile (designated as *Opuntia* polysaccharide fractions C-I, C-II, C-III, and C-IV) (Fig. 1A). Each of the four fractions contained at least 96% carbohydrate and ≤2% protein (Table 1). Negligible levels of protein were also previously reported in mucilage of *O. ficus-indica* [30].

![Figure 1](https://example.com/figure1.png)

**Figure 1** Preparative and analytical size-exclusion chromatography of *Opuntia* polysaccharides. Panel A. The crude *Opuntia* polysaccharide extract was fractionated using Sepharose 6B column chromatography, and the eluate was combined to obtain the four fractions (designated C-I through C-IV). Total carbohydrate content of the fractions was determined as described (detected at 488 nm). Panel B. The *Opuntia* polysaccharide fractions were analyzed by HP-SEC, and eluates were monitored with a refractive index detector, as described. The arrows indicate the peak retention times of the indicated pullulan standards used for calibration.

![Table 1](https://example.com/table1.png)

**Table 1** Biochemical properties of *Opuntia* polysaccharide fractions

<table>
<thead>
<tr>
<th>Polysaccharide fractions</th>
<th>Molecular weight (kDa)</th>
<th>Yariv test</th>
<th>Carbohydrate content (%)</th>
<th>Protein content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-I</td>
<td>733</td>
<td>Negative</td>
<td>96</td>
<td>2</td>
</tr>
<tr>
<td>C-II</td>
<td>550</td>
<td>Negative</td>
<td>99</td>
<td>&lt;1</td>
</tr>
<tr>
<td>C-III</td>
<td>310</td>
<td>Positive</td>
<td>98</td>
<td>&lt;1</td>
</tr>
<tr>
<td>C-IV</td>
<td>168</td>
<td>Positive</td>
<td>98</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

(Sources: [30], [31], [32].)
Very-high-field (600 MHz) $^1$H NMR was used to characterize the structure of native Opuntia polysaccharides (Fig. 2). The spectra from all fractions were similar to each other, suggesting a common backbone structure, and resembled the spectra of polysaccharides isolated from O. ficus-indica [30]. The weak signals present at 3.38–3.45 ppm can be assigned to $\alpha$-rhamnopyranose ($\alpha$-Rha p), while the strong signals at 3.68–3.90 ppm are consistent with the presence of $\beta$-galactopyranose ($\beta$-Gal p) [31]. The signals at 4.05–5.05 ppm are consistent with the presence of $\alpha$-arabinofuranose ($\alpha$-Ara f) and $\alpha$-galacturono-pyranose ($\alpha$-GalA p) residues [31,32].

3.2. Effect of Opuntia polysaccharides on macrophage NO and ROS production

A minimum amount of NO was produced when murine J774.A1 macrophages were incubated with medium alone; whereas, treatment of these cells with any of the four Opuntia polysaccharide fractions resulted in a concentration-dependent increase in NO production (Fig. 3A), with fraction C-I being the most active. Indeed, NO production induced by fraction C-I at 800 μg/ml was comparable to that induced by 100 ng/ml LPS.

In the absence of any treatment, murine J774.A1 macrophages generated very low levels of ROS (Fig. 4, control); whereas, a concentration-dependent enhancement of ROS production was observed in macrophages treated with 25–400 μg/ml doses of each polysaccharide fraction, with fraction C-I being the most active (Figs. 3B and 4). Thus, the individual polysaccharide fractions from O. polyacantha showed a similar pattern with respect to their ability to induce NO and ROS production by murine J774.A1 macrophages (C-I>C-II>C-III>C-IV).

Since endotoxin (LPS) is often a contaminant in biological preparations, we evaluated possible contribution of endotoxin Table 2: Monosaccharide composition of Opuntia polysaccharide fractions

<table>
<thead>
<tr>
<th>Polysaccharide fraction</th>
<th>Galactose (mol%)</th>
<th>Galacturonic acid (mol%)</th>
<th>Xylose (mol%)</th>
<th>Arabinose (mol%)</th>
<th>Rhamnose (mol%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-I</td>
<td>34</td>
<td>15</td>
<td>29</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>C-II</td>
<td>35</td>
<td>15</td>
<td>25</td>
<td>15</td>
<td>10</td>
</tr>
<tr>
<td>C-III</td>
<td>43</td>
<td>19</td>
<td>20</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>C-IV</td>
<td>48</td>
<td>18</td>
<td>17</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>

Monosaccharides were identified and quantified based on TLC analysis of known standards.

Figure 2: $^1$H NMR spectra of Opuntia polysaccharide fractions. Fractions C-I through C-IV were dissolved in D$_2$O, and spectra were recorded at 20 °C.

Figure 3: Effect of Opuntia polysaccharides on murine macrophage ROS and NO production. Murine J774.A1 macrophages were incubated for 24 h with the indicated concentrations of polysaccharide fractions C-I (□), C-II (●), C-III (○), C-VI (♦), or 100 ng/ml LPS (◊, positive control). NO production was quantified by measuring nitrite in the cell-free supernatants (Panel A), and chemiluminescence was monitored using an L-012 detection system, as described (Panel B). Values are the mean±S.D. of triplicate samples from one experiment, which is representative of two independent experiments. Statistically significant differences ($P<0.05$) between untreated cells and cells treated with fractions C-I (†, ‡, †), C-II (†, ‡, †), C-III (†, †), and C-IV (†) are indicated.
to the observed biological activity of *Opuntia* polysaccharides. All fractions caused a similar positive *Limulus* response (data not shown), suggesting polysaccharides present in our fractions were also activating the *Limulus* coagulation cascade (see Discussion). Thus, we evaluated NO production by macrophages treated with *Opuntia* polysaccharide fractions or LPS in the presence and absence of polymyxin B. As shown in Fig. 5, polymyxin B almost completely suppressed macrophage NO production induced by LPS (>90% inhibition). In comparison, polymyxin B only slightly reduced responses induced by the individual *Opuntia* polysaccharide fractions (∼25% inhibition), suggesting that the observed responses were not due to LPS contamination. The small amount of inhibition observed with polymyxin B treatment is likely due to nonspecific effects of this compound on the cells (see Discussion). To eliminate this artifact, we pretreated *Opuntia* polysaccharide samples (fraction C-I) by elution through a column of endotoxin-removing gel so that the cells were not exposed directly to polymyxin B and found that treated polysaccharides were just as active as untreated samples. This control, which is present in subsequent assays, further demonstrates LPS contamination is not responsible for the responses observed (see Figs. 6–10).

Discussion. To eliminate this artifact, we pretreated *Opuntia* polysaccharide samples (fraction C-I) by elution through a column of endotoxin-removing gel so that the cells were not exposed directly to polymyxin B and found that treated polysaccharides were just as active as untreated samples. This control, which is present in subsequent assays, further demonstrates LPS contamination is not responsible for the responses observed (see Figs. 6–10).

To determine whether human phagocytes also responded to *Opuntia* polysaccharides, we analyzed their ability to activate ROS production by human MonoMac6 macrophages. *Opuntia* polysaccharides stimulated low levels of ROS production by MonoMac6 cells in a concentration-dependent manner (Fig. 6A). Furthermore, *Opuntia* polysaccharides significantly enhanced PMA-mediated activation of ROS production by MonoMac6 cells (Fig. 6B), suggesting these polysaccharides can prime cells similar to LPS. Note, however, that pretreatment of fraction C-I with endotoxin-removing gel had no effect on its activity, indicating
that the effect was not due to LPS contamination (Fig. 6). Overall, these data verify that *Opuntia* polysaccharides are not specific for murine phagocytes and can also activate human phagocytes.

3.3. Effect of *Opuntia* polysaccharides on macrophage TNF-α and IL-6 production

A number of immunomodulatory compounds can regulate cytokine production. Thus, we analyzed the effects of *Opuntia* polysaccharide fractions on macrophage TNF-α and IL-6 production. Untreated murine J774.A1 and human MonoMac6 macrophages produced very little TNF-α and IL-6; whereas, incubation of these cells with the *Opuntia* polysaccharide fractions significantly enhanced TNF-α and IL-6 production in a concentration-dependent manner (Figs. 7 and 8). These responses were quite robust, and the levels of TNF-α induced by 20–40 μg/ml of fraction C-I were comparable to those induced by 50 ng/ml LPS in both murine and human cells. Furthermore, cytokine production induced by fraction C-I was essentially the same for untreated samples and samples pretreated with endotoxin-removing gel, further demonstrating that LPS is not responsible for the observed effects (Figs. 7 and 8B).

The effect of *Opuntia* polysaccharides on macrophage TNF-α production was also confirmed in human primary monocyte-derived macrophages. All fractions induced TNF-α production by monocyte-derived macrophages (Fig. 9). Again, responses were not significantly different between untreated and polymyxin gel-pretreated samples of fraction C-I. Thus, these results indicate that *Opuntia* polysaccharides induce a significant up-regulation of TNF-α and IL-6 synthesis in murine as well as in human macrophages.

3.4. Effect of *Opuntia* polysaccharides on nuclear factor kappa B (NF-κB) activation

To evaluate signaling pathways involved in the immunomodulatory activity of *Opuntia* polysaccharides, we utilized a transcription...
factor-based bioassay for NF-κB activation in human THP-1 monocytes. All fractions, including fraction C-I pretreated with endotoxin-removing gel, dose-dependently stimulated NF-κB directed alkaline phosphatase expression in THP-1 human monocytes (Fig. 10), indicating that these polysaccharides potently activate the NF-κB pathway. Indeed, alkaline phosphatase release induced by fractions C-I and C-II at concentration of 80 μg/ml was even greater than that induced by 100 ng/ml LPS. Note also that the activity of untreated fraction C-I was not significantly different than that of fraction C-I pretreated with endotoxin-removing gel (Fig. 10).

3.5. Effect of Opuntia polysaccharides on cell viability

Although our functional assays suggested that Opuntia polysaccharides were relatively non-toxic, we evaluated the potential cytotoxic effect of the polysaccharides to determine if the results might be influenced by background toxicity. Using a cytotoxicity assay, we determined that none of the fractions significantly affected proliferation/viability of J774.A1 cells over the entire concentration range of polysaccharide fractions tested (100–800 μg/ml), verifying that these polysaccharides were not toxic to these cells (Fig. 11).

4. Discussion

Since ancient times, fruits and stems of different species of the genus Opuntia have been used in remedies for treating a number of medical problems. For example, the stems of O. polyacantha have been used to treat infections and wounds [33], which suggests that O. polyacantha extracts might have immune-enhancing effects. Despite the wide-spread use of O. polyacantha, little is known regarding the active components responsible for its therapeutic properties. Previous studies indicated that some of the biological properties of O. polyacantha were due to the presence glycoproteins and low-molecular weight compounds, such as flavonoids, proanthocyanidins, carotenoids, betalains, β-sterols, and α-pyrones [13,15,18–22]. In the present report, we provide evidence suggesting that polysaccharides of O. polyacantha have potent immunomodulatory properties that can enhance monocyte/macrophage function and suggest that this may contribute, at least in part, to the therapeutic potential of O. polyacantha tissues.
We isolated four polysaccharide fractions from the stems of *Opuntia polyacantha* and provided initial structural and pharmacological characterization. Average molecular weights of the fractions were 733, 550, 310, and 168 kDa for fractions C-I through C-IV, respectively, and sugar composition was similar to the monosaccharide composition of polysaccharides isolated from another cactus species, *O. ficus-indica* [30,34]. Analysis of the fractions using the Yariv test showed that two relatively high molecular weight *Opuntia* polysaccharide fractions C-I and C-II lacked arabinogalactan; whereas, the other fractions (C-III and C-IV) tested positive for the presence of type II arabinogalactan (Table 1). Type II arabinogalactan has a β-(1,3)-linked galactan backbone with side chains containing arabinose and galactose residues and have been reported to possess a variety of biological activities [4]. Indeed, in polysaccharide fractions C-III and C-IV, galactose was the dominant monosaccharide and represented >40 mol% of the total sugars in these fractions. However, the most potent biological properties were associated with fractions C-I and C-II. Thus, type II arabinogalactans do not appear to be the main structures responsible for macrophage-activating properties of the *Opuntia* polysaccharides. This observation is supported by our previous studies on other plant-derived polysaccharides [35,36].

Macrophages play critical roles in host defense, including phagocytosis of pathogens and apoptotic cells, production of cytokines, and proteolytic processing and presentation of foreign antigens (reviewed in [37]). Thus, the identification of agents that can modulate macrophages is of significant interest. Indeed, a variety of plant polysaccharides have been reported to exhibit beneficial pharmacological effects via their ability to modulate macrophage function (reviewed in [6]). In this study, all *Opuntia* polysaccharides contained potent macrophage immunomodulatory activity, as demonstrated by induction of effector molecules, such as NO, ROS, and cytokines. In addition, treatment with *Opuntia* polysaccharides before PMA resulted in a significantly enhanced response, which is indicative of a priming effect. Many immunomodulatory compounds, including LPS, can prime phagocytes for enhanced ROS production, and it is generally thought that priming plays a key role in the host defense process and may be essential for host survival against microbial pathogens [38]. Note that macrophage ROS production plateaued or even decreased slightly at higher polysaccharide concentrations, especially with murine macrophages. Based on our previous studies showing that Artemisia polysaccharides can scavenge ROS [36], we suggest that this effect may be due, at least in part, to ROS scavenging by residual *Opuntia* polysaccharides present in the wells (<10% of the initial concentrations), since the cells were not washed when the medium was replaced with fresh medium containing the detection reagents. Indeed, we found that all of the *Opuntia* polysaccharide fractions exhibited ROS scavenging activity in an enzymatic ROS-generating system, with the high molecular weight fractions C-I and C-II being the most active (data not shown). Thus, wells treated with the highest polysaccharide concentrations would have the greatest residual polysaccharide concentrations and the greatest ROS scavenging effect, which would explain the apparent plateau in ROS production. Nevertheless, further studies are needed to fully evaluate the ROS scavenging properties of *Opuntia* polysaccharides.

The macrophage immunomodulatory activity of *Opuntia* polysaccharides seems to be positively correlated with the average molecular weight of the polysaccharides, with higher molecular weight fractions being the most active. This may be a common feature of plant polysaccharides that modulate macrophage function, as we found previously that biological activity correlated with molecular weight for polysaccharides isolated from *Juniperus scopolorum* [39], *Tanacetum vulgare* [35], and *Artemisia tripartite* [36]. Likewise, biological activity of mannans from *Aloe vera* [40] and β-glucans from mushrooms [41] was found to correlate with increased size. Overall, these observations suggest that *Opuntia* and other plant-derived polysaccharides may activate macrophages via receptor(s) or other surface structures, although the nature of these surface targets is currently unknown. The potent macrophage-stimulatory effect of high molecular weight polysaccharides may also be related to their highly repetitive structures, which could cross-link receptors or other membrane targets in a multivalent fashion [40]. Clearly, further studies are necessary to identify the cellular target of these polysaccharides and understand the relationship between their molecular weight and biological activity. For example, it is not clear how such plant-derived polysaccharides are metabolized in the gut and whether this affects their bioavailability. Traditionally, extracts from *Opuntia* species have been used topically for treatment of wounds and burns, as well as internally for a variety of diseases [10,11]. Thus, bioavailability of various components of *Opuntia* extracts may vary, depending on the site of application and other metabolic processes.

A number of plant-derived polysaccharides have been shown to induce production of TNF-α and/or IL-6 in macrophages (e.g., see [6,35,36]). However, this is the first report to demonstrate induction of these cytokines by *Opuntia* polysaccharides. Among the proinflammatory cytokines, IL-6 is one of the most important mediators of fever and the acute-phase response [42]. TNF-α also plays an important role as a key cytokine in immune and inflammatory reactions. TNF-α has direct in vitro and in vivo cytotostatic and cytocidal effects and, together with IL-6, is also considered as a major immune and inflammatory mediator [42]. One of the most prominent characteristics of TNF-α is its ability to cause apoptosis of tumor-associated endothelial cells, resulting in tumor necrosis [43]. TNF-α also plays a pivotal role in host defense and can act on monocytes and macrophages in an autocrine manner to enhance various function responses and induce the expression of a number of other immunoregulatory and inflammatory mediators [44]. Furthermore, TNF-α has been reported to exert in anti-influenza effect, which is greater than that of γ- and α-interferons [45]. In addition to enhancement of cytokine production, *Opuntia* polysaccharides also activated macrophage NO and ROS production. These reactive oxidants play key roles in host defense and other physiological processes and are also involved in the regulation of apoptosis and immune homeostasis (reviewed in [46,47]). Overall, our data suggest modulation of the production of cytokines and reactive oxygen/nitrogen species by macrophages likely contributes to the therapeutic effects of *O. polyacantha* extracts and provides further evidence that the *Opuntia* polysaccharides have potent immunomodulatory properties.
Opuntia polysaccharides potently activated transcription factor NF-κB in THP-1 human monocytes. Activation of NF-κB controls multiple genes in phagocytes, and target genes regulated by NF-κB include proinflammatory cytokines, chemokines, inflammatory enzymes, adhesion molecules, receptors, and inhibitors of apoptosis (reviewed in [48]). Therefore, the ability to activate phagocyte NF-κB signaling provides further evidence that Opuntia polysaccharides possess immunomodulatory properties and confirms that human phagocytes respond to these compounds. In support of this finding, a number of polysaccharides have been reported previously to activate NF-κB. For example, high molecular weight polysaccharides from Aloe barbadensis increased NF-κB-directed luciferase expression in THP-1 cells [49]. Likewise, high M₄ polysaccharides from Spirulina platensis, Aphanizomenon flos-aquae, and Chlorella pyrenoidosa were found to activate NF-κB, leading to increased immune cytokine mRNA levels [50]. Clearly, activation of NF-κB plays a critical role in the transcriptional regulation of TNF-α and inducible NO synthase (iNOS) genes [51]. In addition, TNF-α [52] and NO [53] generated upon cell activation can further activate the NF-κB pathway. Thus, it is reasonable that Opuntia polysaccharide fractions dose-dependently stimulated macrophage TNF-α, IL-6 and NO production and also activated NF-κB in a similar pattern. In future studies, it will be interesting to determine whether these polysaccharides enhanced the production of proinflammatory mediators (e.g., TNF-α and NO) through activation of NF-κB and/or vice versa.

Endotoxin (LPS) is a known immunomodulator and is often a contaminant in biological preparations. Therefore, a number of approaches were utilized to evaluate and prevent possible LPS contamination. Since the crude extract used for isolation of the Opuntia polysaccharide fractions was prepared from intact, uninjured green stems of O. polyacantha after washing in ultrapure water, bacterial contamination of the stems was unlikely. Furthermore, isolation was conducted under conditions that minimized the possibility of bacterial contamination, and the fractions were filtered through 0.22 μm filters prior to biological assays. However, further analyses were performed to directly evaluate this issue. Initially, we evaluated Opuntia polysaccharide fractions using the Limulus-based endotoxin assay. We found that all four fractions caused a positive Limulus response. This is not surprising, as a number of plant polysaccharides have been shown to react in the Limulus assay, presumably through their ability to mimic LPS [39,54,55]. Thus, we evaluated the effects of polymyxin B on polysaccharide activity. This compound can bind to and inactivate LPS, and we found that inclusion of polymyxin B only had little effect on macrophage NO production induced by Opuntia polysaccharides, while it completely inhibited activation by LPS. The partial inhibition of the polysaccharide activity observed after polymyxin B treatment is likely due to nonspecific effects of this agent. For example, polymyxin B has been reported to cause down-regulation of phospholipid-sensitive Ca²⁺-dependent protein kinase [56] and inhibition of iNOS gene expression and cytokine production in murine macrophages [57]. In addition, polymyxin B is a surface-active peptide that may interact with sites on the plasma membrane functionally related to the targets of plant polysaccharides [58]. Polymyxin B is a cationic peptide that could also directly bind to and/or neutralize active anionic groups in plant polysaccharides. Because of these issues, we performed additional control experiments using polysaccharides eluted through a column of endotoxin-removing gel and showed that polysaccharides treated with endotoxin-removing gel were as active as untreated samples. Therefore, we are confident that the biological activity in our samples is due to the Opuntia polysaccharides and not a contaminating artifact.

In summary, the present study demonstrates that high molecular weight polysaccharides from the stems of O. polyacantha are not cytotoxic and activate macrophages, resulting in modulation of NO, ROS, and cytokine production. Our data provide a molecular basis to explain at least part of the beneficial therapeutic effects reported for extracts of O. polyacantha, and suggest that macrophage stimulation by the Opuntia polysaccharides might enhance resistance to infection. Further studies are now in progress to determine which receptor(s) are essential to the expression of the various immunomodulatory effects ascribed to Opuntia polysaccharides and other structural features conferring biological activity.

Acknowledgements

We would like to thank Jerry and Linda Iverson (Big Timber, MT) for providing prickly pear cactus from their ranch and Dr. Scott Busse, Montana State University, Bozeman, MT for the help in running NMR samples. This work was supported in part by the Department of Defense grant W9113M-04-1-0001, National Institutes of Health grants P20 RR-020185 and U54 AI-065357, National Institutes of Health contract HHSN266200400009C, an equipment grant from the M.J. Murdock Charitable Trust, and the Montana State University Agricultural Experimental Station. The U.S. Army Space and Missile Defense Command, 64 Thomas Drive, Frederick, MD 21702 is the awarding and administering acquisition office. The content of this report does not necessarily reflect the position or policy of the U.S. Government.

References


